Phylogenetic Positions of the Enigmatic Asiatic Fern Genera *Diplaziopsis* and *Rhachidosorus* from Analyses of Four Plastid Genes

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Abstract.—Nucleotide sequences from four plastid genes (*rbcL, atpB, atpA, rps4*) were used to infer relationships of *Diplaziopsis* and *Rhachidosorus*. The phylogenetic positions of these two Asian fern genera have been debated, and neither had been included in the most recent global molecular systematic studies of ferns. Our four plastid gene sequence analyses supported a sister relationship between *Diplaziopsis, Rhachidosorus* and the North American *Homalosorus*, the monophyletic group of the newly-examined genera is an early diverging lineage of Woodsiaceae, and far away from athyrioid ferns. The inferred relationships of *Diplaziopsis* and *Rhachidosorus* are not consistent with most recent treatments, while, some synapomorphic characteristics are shared with these two genera. Further studies on more morphological characters and gametophytes of these two genera are needed to test these relationship hypotheses.

The fern family Woodsiaceae, as circumscribed in the most recent familial classification (Smith *et al.*, 2006), comprises about 15 genera and more than 700 species distributed mainly from tropical America to Old World temperate, which is characterized by monomorphic or nearly monomorphic leaves and vascular anatomy (Tryon and Tryon, 1982). The family exhibits an extensive dysploid series of base chromosome numbers, ranging from 31 to 42, mostly $x = 40, 41$, also 31 (*Hemidictyum*), 33, 38, 39 (*Woodsia*), and 42 (*Cystopteris*) (Smith *et al.*, 2006). The monophyly of Woodsiaceae of Smith *et al.* (2006) is lacking in all broad analyses (Hasebe *et al.*, 1995; Sano *et al.*, 2000; Schneider *et al.*, 2004; Schuettpelz and Pryer, 2007). The more recent fern global phylogenetic analyses showed Woodsiaceae of Smith *et al.* (2006) consists of four well-supported clades: together, *Cystopteris* and *Gymnocarpium* are sister to the rest of eupolypods II; *Hemidictyum* is sister to the asplenioid ferns; and *Woodsia* is sister to a large clade of onocleoid, blechnoid, and athyrioid ferns (Schuettpelz and Pryer, 2007). This is the most inclusive analysis of leptosporangiate fern relationships conducted to date, in which three plastid genes (*rbcL, atpA, atpB*) from 400 leptosporangiate fern species were utilized. However, the taxomically problematic genera *Diplaziopsis, Homalosorus* and

Rhachidosorus were not included in this groundbreaking study, and the phylogenetic affinities of these taxa are unclear. Smith *et al.* (2006) tentatively placed these genera in the Woodsiaceae and suggested, "further sampling will likely shed additional light on this subject, and the recognition of several additional families may be warranted."

Diplaziopsis is a problematic genus, which has undergone many systematic changes. Bower (1928) treated it provisionally along with his "Asplenoid ferns", while Christensen (1938) revised his opinion to give it a generic status and treats as a group of ferns with areolate veins under *Diplazium* Sw.. Copeland (1947) defined *Diplaziopsis* by its pinnate leaves, thin lamina texture, anastomosing veins, and sausage-like sori, comprising two species (*D. javanica* and *D. cavaleriana*). Ching (1964a) added two new species from China to *Diplaziopsis*, which were later emended into three species in the flora of China (i.e., *D. javanica*, *D. cavaleriana*, and *D. brunoniana*) (Chu *et al.*, 1999). In geographical distribution, *Diplaziopsis* is essentially an Old World genus mainly from Eastern and Southeastern Asia (Ching, 1964a).

The monotypic genus *Homalosorus* was established by Pichi Sermolli (1973) with *H. pycnocarpos* distributed only in the temperate eastern North America, but other authors have included it in the genus *Athyrium* Roth (Kramer and Kato, 1990) or the genus *Diplazium* (Tryon and Tryon, 1982; Kato and Iwatsuki, 1983). A relationship between *H. pycnocarpos* and *Diplaziopsis* (Tryon and Tryon, 1973; Kato and Iwatsuki, 1983) has been suggested from their similar pinna shape, rachis-grooving, indusia, and spores, although they have different lamina apex, venation, and chromosome numbers (Kato and Darnaedi, 1988; Price, 1990). The sister relationship between *H. pycnocarpos* and *Diplaziopsis* has been supported by *rbcL* (Sano *et al.*, 2000) and *rbcL+rps4* trees (Wei *et al.*, 2010), while a previous *trnL-F* study lent support to the placement of *Diplaziopsis cavaleriana* in *Diplazium* (Wang *et al.*, 2003).

Previously, plants here recognized as *Rhachidosorus* have been included in either *Athyrium* (Tagawa, 1936) or *Diplazium* (Kato, 1977; Kramer and Kato, 1990), but Ching (1964b) later separated those plants into the genus *Rhachidosorus* from South-east Asia, and determined that the genus consists of eight species. The genus *Rhachidosorus* differs from both *Athyrium* and *Diplazium* (or rather *Allantodia* R. Br.; most *Diplazium* species in China have been placed in *Allantodia*) in having thick creeping rhizomes, the scales near the base of stipe and, above all, in the narrow semilunate sori and indusia of the asplenioid type, which are never diplazioid nor athyrioid, and in the spore morphology (Ching, 1964b). Based on previous *rbcL* (Sano *et al.*, 2000) and *trnL-F* analyses (Wang *et al.*, 2003), *Rhachidosorus* does not cluster with either *Athyrium* or *Diplazium* but occupies a position isolated from the other taxa in the eupolypods II; such a conclusion was suggested by Ching (1964b; 1978).

In this study, we use more DNA sequence data from four plastid genes (*rbcL*, *atpB*, *atpA*, *rps4*) to make comparisons with the previous studies, to investigate the phylogenetic relationships of *Diplaziopsis*, *Homalosorus*, and *Rhachidosorus* to other ferns of eupolypods II, and specifically to address whether they belong within athyrioid ferns.

Materials and Methods

Taxon Sampling.—In order to make comparisons with other studies (Sano *et al.*, 2000; Schuettpelz and Pryer, 2007), we assembled three data matrices (Table 2), all of which included newly generated sequences and sequences obtained from GenBank. A total of 98 new sequences were generated for this study, the corresponding voucher specimens have been deposited in the Herbarium of the Yunnan University (PYU). Taxa, vouchers, and accession numbers are provided in Table 1. The first data matrix consisted of 59 *rbcL* sequences, of which 24 were newly generated. The second matrix comprised *rbcL*, *atpB* and *atpA* sequences of 59 taxa, which included 22 *atpB* and 20 *atpA* sequences newly generated in this study plus additional sequences from GenBank. The third matrix comprised *rbcL*, *atpB*, *atpA* and *rps4* sequences of 59 taxa, which included the three sequences of 59 taxa from the second matrix and 32 *rps4* newly generated in this study plus additional sequences from GenBank. Those taxa with incomplete sequences were included in the analyses of the combined data, and the unsequenced fragments were coded as missing data. In order to investigate the phylogenetic relationships of *Diplaziopsis*, *Homalosorus*, and *Rhachidosorus* to other genera, our sampling included 14 of 15 recognized genera in Woodsiaceae as treated by Smith *et al.* (2006). The two previously unincluded Asian genera in the study of Schuettpelz and Pryer (2007), *Diplaziopsis* and *Rhachidosorus*, are represented by two or more species, with each species represented by one or more specimens. In addition, we examined species of Aspleniaceae, Blechnaceae, Onocleaceae, and Thelypteridaceae, which were all included with the Woodsiaceae in the eupolypods II clade of Smith *et al.* (2006). Following the previously published molecular systematic studies of leptosporangiate ferns (Schuettpelz and Pryer, 2007), in which it is well established that eupolypods II is most closely related to eupolypods I, we selected *Drynaria rigidula*, *Dryopteris uniformis* and *Polypodium vulgare* as outgroups.

DNA extraction, gene amplification, and sequencing.—Total genomic DNA was extracted from 2 g of fresh or 1 g of silica gel dried leaves using the CTAB procedure (Doyle and Doyle, 1987). The selected DNA regions were amplified with standard polymerase chain reaction (PCR). The protocols used to amplify four genes were identical and followed Li *et al.* (2004). For information on amplification and sequencing primers, see Table 3.

Sequence analysis.—The obtained sequences have been assigned GenBank accession numbers (Table 1). Alignments of all sequences were performed using Clustal X (Thompson *et al.*, 1997) and subsequently edited manually in BioEdit (Hall, 1999). There were no insertions or deletions (indels) in the protein-coding sequence alignments. Indels were introduced into the alignment of *rps4–trnS* spacer region, in which ambiguously aligned regions were excluded from all analyses. Phylogenetic analyses were investigated by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods in PAUP* 4.0b10 (Swofford, 2002), PHYML 2. 4. 3 (Guindon and Gascuel, 2003), and MrBayes 3. 1. 2 (Ronquist and Huelsenbeck, 2003). For MP

analysis, unweighted analyses were performed by heuristic searches with tree-bisection-reconnection (TBR) branch swapping, the MulTrees in effect, steepest descent off using 1000 random taxon-addition replicates, and one tree held at each step during stepwise addition. Bootstrap analyses (Felsenstein, 1985) were conducted to examine the relative level of support for individual clades on the cladograms of each search (MPBS), using 500 bootstrap replicates and the same tree search procedure as described above. For the ML and BI analyses, the best-fitting model of sequence evolution for each data was identified with the Akaike Information Criterion in Modeltest 3.07 (Posada and Crandall, 1998). The SYM+I+G model was selected for the *rbcL* data set, and the GTR+I+G model was selected for the combined data sets (Table 2). Once the best sequence evolution model was determined, the ML analysis was performed for each data set, the parameters such as base-composition, Gamma-shape, and ratio of invariable sites were also estimated during each ML analysis. Nodal robustness on the ML tree was estimated by the nonparametric bootstrap (500 replicates, MLBS). BI was conducted using MrBayes 3.1.2 with appropriate evolutionary models determined as described above and the default priors. We ran two concurrent analyses, each with four chains of the Markov chain Monte Carlo, sampling one tree every 100 generations of $2 \times 1,000,000$ generations, starting with a random tree. The first 25% of the samples (5000 trees) were discarded as "burn-in". At this point, the standard deviation of split frequencies was <0.01, indicating that convergence to a stationary distribution had been achieved. The posterior probability (PP) was used to estimate nodal robustness.

RESULTS

The alignment length and the number of included characters for the three data sets are presented in Table 2. The aligned *rbcL* matrix contained 1308 characters, of which 417 were variable. MP, ML and BI analyses of *rbcL* matrix resulted in nearly identical topologies, with several minor differences at the genus level (results not shown). Strong support was lacking along the backbone of the *rbcL* tree. The 50% majority-rule consensus tree revealed that eupolypods II fall into nine lineages (Fig. 1): athyrioids (Woodsiaceae I), Blechnaceae + Onocleaceae, Woodsiaceae II (*Woodsia, Prowoodsia* and *Cheilanthopsis*), Thelypterdiaceae, Woodsiaceae III (*Cystopteris, Acystopteris* and *Gymnocarpium*), Woodsiaceae IV (*Diplaziopsis* and *Homalosorus*, in shadow in Fig. 1), Woodsiaceae V (*Rhachidosorus*, in shadow in Fig. 1), Aspleniaceae, and Woodsiaceae VI (*Hemidictyum*). In the *rbcL* tree, all four *Rhachidosorus* specimens are united in a single clade; two species of *Diplaziopsis* form another monophyletic clade with *Homalosorus*; the three genera of *Woodsia, Prowoodsia* and *Cheilanthopsis* (Woodsiaceae II) are united in a single clade; and the three genera of *Cystopteris, Acystopteris* and *Gymnocarpium* (Woodsiaceae III) are united in another one. All four clades are isolated from other genera in the family. *Hemidictyum* in Woodsiaceae is sister to Aspleniaceae with low support (PP = 0.90 and MLBS, MPBS < 50%); the

TABLE 1. Taxa Examined and GenBank Accession Numbers.

Family and Species	rbcL	atpB	atpA	rps4[a]	Locality and Voucher[b]
Outgroups					
Drynaria rigidula (Sw.) Bedd.	EF463247	EF463493	EF463811	AY529188	Zhejiang, SG LU/FH44
Dryopteris uniformis (Makino) Makino	EF463183	EF463395	EF463679	**JN168069**	
Polypodium vulgare L.	AB044899	EF463510	EF463841	EF551081	
Aspleniaceae					
Asplenium auritum Sw.	EF463146	EF463327	EF463591	AY549759	
Asplenium normale D. Don	EF463152	EF463337	EF463601	AY549784	
Asplenium trichomanes L.	EF463157	EF463349	EF463613	EF645629	
Hymenasplenium cheilosorum (Kunze ex Mett.) Tagawa	AB014704	EF463350	EF463614	AY549757	
Hymenasplenium unilaterale (Lam.) Hayata	EF452140	EF452020	EF452078	AY459170	
Blechnaceae					
Blechnum gracile Kaulf.	EF463158	EF463351	EF463615	AF313606	
Blechnum occidentale L.	U05910	U93838	EF452080	AF533868	
Brainea insignis (Hook.) J. Sm.	AF533870	**JN168027**		**JN168070**	Yunnan, SG LU/YY4
Struthiopteris eburnea (Christ) Ching	**JN168003**	**JN168028**		**JN168071**	Hubei, SG LU/Z1
Woodwardia prolifera Hook. et Arn.	AY137666	**JN168029**	**JN168049**	**JN168072**	Fujiang, SG LU/WY17
Woodwardia virginica (L.) Sm.	AY137660	EF463359	EF463623	AF533857	
Onocleaceae					
Onoclea sensibilis L.	U62034	EF463488	EF463793	AF425159	
Thelypteridaceae					
Macrothelypteris torresiana (Gaud.) Ching	EF463277	EF463533	EF463873	AF425172	
Pseudophegopteris tibetana Ching et S. K. Wu	**JN168004**	**JN68030**	**JN168050**	**JN168073**	Xizang, SG LU/XZ72
Thelypteris abrupta (Desv.) Proctor	EF463280	EF463536	EF463876		
Thelypteris palustris Schott	U05947	AY612713	EF452127	AF425189	

TABLE 1. Continued.

Family and Species	rbcL	atpB	atpA	rps4[a]	Locality and Voucher[b]
Woodsiaceae					
Acystopteris japonica (Luerss.) Nakai	JN168005	JN168031	JN168051	JN168074	Sichuan, SG LU/X7
Acystopteris tenuisecta (Bl.) Tagawa	JN168006	JN168032	JN168052	JN168075	Yunnan, SG LU/DL2
Athyrium distentifolium Tausch ex Opiz	EF463304	EF463560	EF463901	AF425152	
Athyrium filix-femina (L.) Roth	U05908	EF463561	EF463902		
Athyrium otophorum (Miq.) Koidz.	EF463305	EF463563	EF463904	JN168076	Sichuan, SG LU/X8
Athyrium niponicum (Mett.) Hance	D43891	EF463562	EF463903	JN168077	Zhejiang, SG LU/FH25
Athyrium yokoscense (Franch. & Sav.) Christ	D43893	EF463564	EF463905	JN168078	Jiangxi, SG LU/JX51
Cheilanthopsis elongata (Hook.) Cop.	JN168007	JN168033	JN168053	JN168003	Yunnan, SG LU/JU33
Cheilanthopsis indusiosa (Christ) Ching	JN168008	JN168034	JN168054	JN168079	Yunnan, SG LU/JU34
Cornopteris decurrenti-alata (Hook.) Nakai	D43897	EF463565	EF463906	JN168080	Zhejiang, SG LU/P9
Cystopteris fragilis (L.) Bernh.	JN168009	JN168035	JN168055	JN168003	Xizang, SG LU/XZ88
Cystopteris kansuana C. Chr.	JN168010	JN168036		JN168081	Xizang, SG LU/XZ89
Cystopteris moupinensis Franch.	JN168011			JN168082	Yunnan, SG LU/JC18
Cystopteris pellucida (Franch.) Ching ex C.Chr.	JN168012	JN168037	JN168056	JN168083	Yunnan, SG LU/JU29
Deparia boninicola (Nakai) M. Kato	D43899	EF463566	EF463907	JN168084	
Deparia lancea (Thunb.) Fraser-Jenk	EF463306	EF463567	EF463908	JN168085	
Deparia petersenii (Kunze) M. Kato	JN168013	EF463568	EF463909	AF425153	Hainan, SG LU/V23
Deparia unifurcata (Baker) M. Kato	EF463307	EF463569		JN168086	Sichuan, SG LU/CQ11
Diplaziopsis brunoniana (Wall.) W. M. Chu	JN168014	JN168038	JN168057	JN168087	Yunnan, SG LU/BN10
Diplaziopsis cavaleriana (Christ) C. Chr.	JN168015	JN168039	JN168058	JN168088	Sichuan, SG LU/CQ1
Diplaziopsis cavaleriana (Christ) C. Chr.	JN168016		JN168059	JN168089	Yunnan, SG LU/XCB13
Diplazium bombonasae Rosenst.	EF463308	EF463570	EF463911		
Diplazium centripetale (Baker) Maxon	EF463309	EF463571	EF463912		
Diplazium conterminum H. Christ	JN168017	JN168040	JN168060	JN168090	Sichuan, SG LU/X37
Diplazium cristatum (Desr.) Alston	EF463310	EF463572	EF463913		

TABLE 1. Continued.

Family and Species	rbcL	atpB	atpA	rps4[a]	Locality and Voucher[b]
Diplazium dilatatum Blume	EF463311	EF463573	EF463914	JN168091	Yunnan, SG LU/B49
Diplazium hachijoense Nakai	EF463312	EF463574	EF463915	JN168092	Zhejiang, SG LU/P14
Diplazium legalloi Proctor	EF463313	EF463575	EF463916		
Diplazium plantaginifolium (L.) Urb.	EF463314	EF463576	EF463917		
Diplazium proliferum (Lam.) Thouars	EF463315	EF463577	EF463918		
Diplazium wichurae (Mett.) Diels	JN168018	EF463579	EF463920	JN168093	Guangxi, SG LU/GX56
Gymnocarpium jessoense (Koidz.) Koidz.	JN168019	JN168041	JN168061	JN168094	Yunnan, SG LU/K18
Gymnocarpium oyamense (Baker) Ching	JN168020	JN168042	JN168062	JN168095	Hubei, SG LU/Z7
Hemidictyum marginatum (L.) C. Presl	EF463318	EF463581	EF463922		
Homalosorus pycnocarpos (Spreng.) Pic. Ser.	AB021722			AF425154	
Protowoodsia manchuriensis (Hook.) Ching	JN168021	JN168043	JN168063	JN168096	Zhejiang, SG LU/P28
Rhachidosorus blotianus Ching	JN168022	JN168044	JN168064	JN168097	Yunnan, SG LU/B46
Rhachidosorus consimilis Ching	JN168023	JN168045	JN168065	JN168098	Yunnan, SG LU/J21
Rhachidosorus consimilis Ching	JN168024	JN168046	JN168066	JN168099	Yunnan, SG LU/MLP25
Rhachidosorus consimilis Ching	JN168025	JN168047	JN168067	JN168100	Yunnan, SG LU/YY33
Woodsia polystichoides Eaton	JN168026	JN168048	JN168068	AF425147	Guizhou, SG LU/FJS5

Note. Circumscription of family and genera follows Smith et al. (2006). Accession numbers in boldface type for newly generated sequences.
[a] In the table, rps4 and rps4-trnS IGS are given together because they are submitted to GenBank as a continuous sequence.
[b] Voucher information for newly generated sequences.

TABLE 2. Statistics for the Three Data Sets Analyzed in This Study.

Data set	Included taxa	Alignment length	Variable characters	Best-fitting model[a]
rbcL	59	1308	417	SYM+I+G
rbcL+atpB+ atpA	52	4092	1045	GTR+I+G
rbcL+atpB+ atpA+ rps4	53	5192	2155	GTR+I+G

[a] As identified with the Akaike Information Criterion in Modeltest.

athyrioids clade is resolved in rbcL trees, but support for this relationship is very low (PP = 0.53 and MLBS, MPBS < 50%).

The combined rbcL, atpB and atpA data matrix included 4092 characters, with 1045 characters that were variable. The results from the three combined sequences showed better resolved and supported inter- and intra-familial relationships than that of rbcL tree (results not shown), especially, Woodsiaceae IV (Diplaziopsis and Homalosorus, shown in shadow in Fig. 1) and Woodsiaceae V (Rhachidosorus, shown in shadow in Fig. 1), the focus of our study, are united in a single clade (PP = 1.00, MLBS=65%, MPBS=71%), so the trees reveal that eupolypods II fall into eight lineages.

The four combined data matrix (rbcL, atpB, atpA and rps4) included 5227 characters, with 2127 characters that were variable. MP, ML and BI analyses from the four combined sequences resulted in nearly identical topologies, with most differences at the statistical support values. Because the resultant topologies for relationships of eupolypods II from each of the datasets were not in conflict with one another, the phylogenetic relationships presented here are based on analyses of the four combined data set. The 50% majority-rule consensus tree resulting from MP, ML and BI analyses of the four combined sequences data set is shown in Fig. 1. These analyses yielded an almost robust phylogeny with the exception of a few nodes. Together, Hemidictyum and Aspleniaceae are sister to the rest of eupolypods II, Hemidictyum is sister to

TABLE 3. Primers Used for Amplifying and Sequencing DNA of This Study.

| DNA region	Primer	5'-3' Primers sequence	Primer source
rbcL	rbcLF1	ATG TCA CCA CAA ACG GAG AC	Li et al., 2004
rbcL	rbcLF631	CCA TTC ATG CGY TGG AGA G	This study
rbcL	rbcLR631	CTC TCC ARC GCA TGA ATG G	This study
rbcL	rbcLR1369	GGA CTC CAC TTA CWA GCT TC	This study
atpB	atpBFwood	ATG AGT GCC ACA GAC GG	This study
atpB	atpBRwood	CCA GGA AGA ATC ATT TG	This study
atpA	AtpARF200	GAA TCK GAT AAT GTT GGG	This study
atpA	AtpAR1140	CAG CCA CCT GTT TCA TAG C	This study
rps4	Rps4F	ATG TCC CGT TAT CGA GGA CC	Li et al., 2006
rps4	trnSR	TAC CGA GGG TTC GAA TC	Souza-Chies et al., 1997 1997 al.,1997
rps4	rps4F	CGA GAG TAA TAC TCA ACA AC	This study
rps4	rps4R	ATG AAT TRT TAG TTG TTG AG	This study
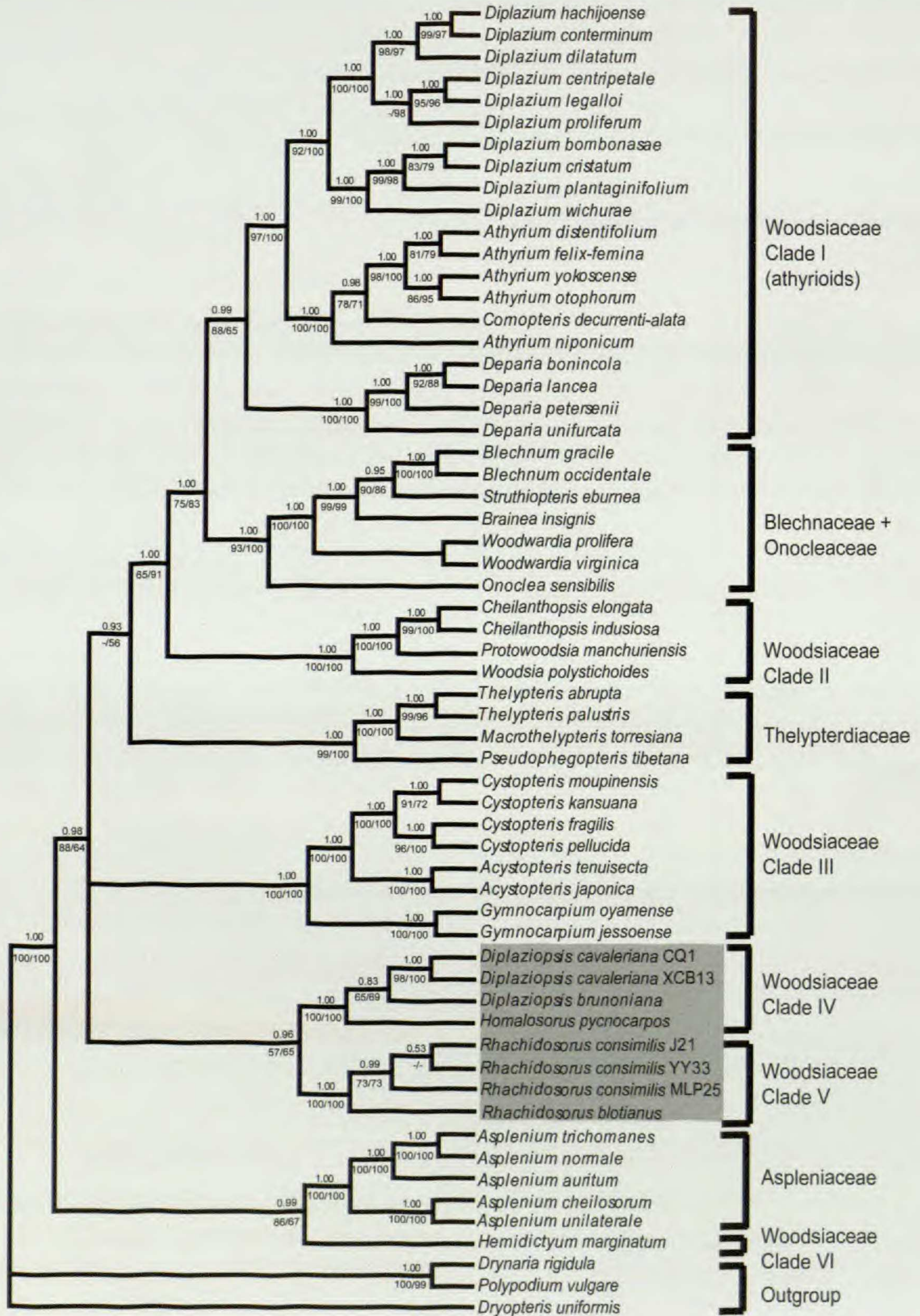
FIG. 1.　Fifty-percent majority-rule consensus tree from Bayesian inference (BI) of the 53-taxon phylogeny based on the combined *rbcL, atpB, atpA*, and *rps4* sequence data. Values above branches are posterior probability from BI; values below branches are bootstrap percentage ≥ 50% from maximum parsimony and maximum likelihood analyses. Six clades within Woodsiaceae are marked in the phylogenetic tree. Taxon names in shadow represent the clade that is newly examined. Family circumscription follows Smith *et al.* (2006).

the asplenioid ferns; then the clade of Woodsiaceae IV and Woodsiaceae V (*Rhachidosorus, Diplaziopsis* and *Homalosorus,* in shadow in Fig. 1); the clade of Woodsiaceae III; and the large clade of athyrioids, Blechnaceae + Onocleaceae, Woodsiaceae II, and Thelypterdiaceae.

DISCUSSION

Phylogenetic relationships of eupolypods II, comparisons with previous studies.—Generally, our phylogenetic results are compatible with previous studies on the relationships among fern genera in eupolypods II. The overall eupolypods II relationships shown in Fig. 1 are not in conflict with the results of Sano *et al.* (2000). Our phylogenetic analyses of multiple chloroplast genes confirmed those results and showed better resolved and supported inter- and intra-familial relationships than that of the *rbcL* tree. With more extensive sampling of Chinese Woodsiaceae, a sister relationship between *Diplaziopsis, Rhachidosorus* and the North American *Homalosorus* was moderately supported by the four chloroplast gene data, and the three genera of *Acystopteris, Cystopteris,* and *Gymnocarpium* were resolved as a monophyletic lineage with strong statistical support. Both were early diverging lineages in eupolypods II, and far away from athyrioid ferns. We mapped the three enigmatic genera (*Diplaziopsis, Rhachidosorus,* and *Homalosorus*) onto the Schuettpelz and Pryer (2007) global fern phylogenetic framework. However, there is a major point of difference between our study and theirs; their phylogeny found the clade of *Cystopteris* and *Gymnocarpium* sister to the rest of eupolypods II, while we found the asplenioid clade (including *Hemidictyum*) sister to the rest of eupolypods II, i.e., the most basal-most lineage of eupolypods II is the clade of asplenioid ferns. It is possible that the sampling of different markers or their combinations caused the topological difference. Plastid DNA is inherited as an intact unit, and differences between trees constructed from separate regions can be due to functional constraints and evolution rates (Wendel and Doyle, 1998). We can correct for both factors by directly combining these separate regions, because combined analyses confidently resolved the conflicts between the single gene analyses, enhanced phylogenetic resolution, and were better supported by morphological information (Gontcharov *et al.*, 2004).

Phylogenetic relationships of Diplaziopsis and Homalosorus.—Cladistic analysis of four plastid gene (*rbcL, atpA, atpB* and *rps4*) sequences provided strong evidence that *Diplaziopsis* and *Homalosorus* form a monophyletic lineage and are clearly separated from *Diplazium.* The relationship agrees with the results of *rbcL* analyses (Sano *et al.* 2000) and a recent study based on *rbcL+rps4* analyses (Wei *et al.*, 2010). *Diplaziopsis* and *Homalosorus* were formerly treated as members of *Diplazium* (Christensen, 1938; Kato, 1977; Kato and Iwatsuki, 1983), with which they shared features such as linear sori, similar stipe base and frond axes (Kato, 1977). *Diplaziopsis* and *Homalosorus* differ from *Diplazium* by lamina simple to once-pinnate, veins anastomosing

with numerous areoles (but not goniopteroid), rachis groove V-shaped, rhizome and roots not black and sclerified (Price, 1990).

In China, Ching had a central role in interpreting the delineation of diplazioid genera (Ching, 1964a, b). He regarded *Diplaziopsis* as one younger offshoot from the great stock of diplazioid ferns, while our four plastid gene sequences analyses revealed that the monophyletic lineage of *Diplaziopsis*, *Rhachidosorus*, and *Homalosorus* diverged earlier than other diplazioid genera, indicating that the lineage may not be a direct derivative from diplazioid ferns as Ching assumed. Consequently, *Diplaziopsis* and *Homalosorus* are morphologically well-defined and should be treated as a separate genus from *Diplazium* as proposed by Sano *et al.* (2000). While living materials of the type species, *Diplaziopsis javanica* (Bl.) C. Chr., and the monotype genus, *Homalosorus pyconcarpos,* are currently unavailable, increased sampling is needed to resolve generic relationships within the clade with more accuracy. With living materials, the morphological and developmental characteristics of the clade can be evaluated in more detail, and then the taxonomic status of this clade and can be revised.

Phytogeography of Diplaziopsis and Homalosorus.—Geographically, *Diplaziopsis* is essentially eastern Asian, while that of *Homalosorus* is eastern North American. The disjunct distributions between eastern North America and eastern Asia are not only demonstrated by many flowering plants (reviewed in Wen, 1999), but also by some ferns (e.g., Tryon and Tryon, 1973; Kato and Iwatsuki, 1983). *Homalosorus pycnocarpos* and species of *Diplaziopsis* have been cited as examples of this by Tryon and Tryon (1973), Kato and Iwatsuki (1983), and Kato and Darnaedi (1988). A vicariance scenario for the disjunct distribution is possible as suggested by Barrington (1993) and Kato (1993). Estimates of divergence times using molecular and palaeontological data to test this hypothesis are currently being performed.

Diplaziopsis, Rhachidosorus, and Homalosorus.—Cladistic analysis of four plastid gene (*rbcL, atpA, atpB* and *rps4*) sequences provide moderate statistical support that *Diplaziopsis, Rhachidosorus,* and *Homalosorus* form a monophyletic lineage (Fig. 1), which has not been recovered in previous single DNA fragment analyses (Sano *et al.,* 2000; Wang *et al.,* 2003). All these three genera share some morphological characteristics with the athyrioid ferns, yet no obvious morphological characters have been identified to support their sister relationship. Morphologically, *Diplaziopsis* and *Homalosorus* have once-pinnate leaves, whereas *Rhachidosorus* has highly divided (bipinnate to tripinnate) blades. *Diplaziopsis* is with reticulated venation, while *Rhachidosorus* and *Homalosorus* with free venation. Above all, the genera differ in their indusium types: *Diplaziopsis* and *Homalosorus* are of very typical diplazioid type and *Rhachidosorus* of the asplenioid one. Some characters of these genera, such as the swelled mature indusium and the basic chromosome number of $x=41$ of *Rhachidosorus* and *Diplaziopsis* (Kato *et al.,* 1992; Nakato *et al.,* 1995), showed some hints for the relationship. Herein, we did not provide strong evidence for their systematic relationships; more studies on morphological and developmental characteristics of these genera are required

that we may be able to identify additional morphological character changes supporting these relationship hypotheses.

The family Woodsiaceae has been variously circumscribed, and its limits are still uncertain (Hasebe *et al.*, 1995; Sano *et al.*, 2000; Schneider *et al.*, 2004; Schuettpelz and Pryer, 2007). Wang *et al.* (2004) divided the Athyriaceae (excluding woodsioid ferns, in their circumscription), by far the largest component in the family, into five subfamilies: Cystopteroideae, Athyrioideae, Deparioideae, Diplazioideae, and Rhachidosoroideae. Because the three enigmatic genera (*Diplaziopsis, Homalosorus,* and *Rhachidosorus*) were not included in the most inclusive analysis of leptosporangiate fern relationships conducted to date (Schuettpelz and Pryer, 2007), and the other two previous studies (Sano *et al.*, 2000; Wang *et al.*, 2003) showed different phylogenetic positions for *Diplaziopsis*, we added the taxa of *Diplaziopsis* and *Rhachidosorus* to our four-gene dataset. As delimited by Smith *et al.* (2006), the monophyly of Woodsiaceae is lacking, because of this uncertainty, Smith *et al.* (2006) believe that further sampling will likely shed additional light on this subject, and the recognition of several additional families may be warranted. Our analyses revealed another lineage in Woodsiaceae of Smith *et al.* (2006), i.e., the *Diplaziopsis-Homalosorus-Rhachidosorus* lineage, which is clearly helpful for Woodsiaceae realignments within the next few years. Because the overall topology of eupolypods II is not yet well-resolved (Schuettpelz and Pryer, 2007) and the clade of *Diplaziopsis-Homalosorus-Rhachidosorus* in this study is only moderately supported, we do not advocate major taxonomic realignments at this time. Within the next few years we expect that increased taxon sampling, combined with additional morphological and molecular studies, will result in a phylogenetically accurate scheme for a better classification of Woodsiaceae.

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